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Award Number: DAMD17-98-1-8494

TITLE: Identification of Prostate Specific Antigens for Immunotherapy Vaccines

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REPORT DATE: February 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2001	Final (1 Aug 98 - 31 Jan 01)

4. TITLE AND SUBTITLE
Identification of Prostate Specific Antigens for Immunotherapy Vaccines

5. FUNDING NUMBERS
DAMD17-98-1-8494

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING-/ MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Progress Report

Introduction

The limiting step in the development of antigen specific vaccines for the immunotherapy of cancer is the identification of tissue or cancer specific antigens capable of activating a strong immune response. Many antigens with the desired specificity have been identified but their function as target antigens in immunotherapy are unknown. For prostate cancer several antigens with a prostate restricted expression pattern, including prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), prostatic acid phosphatase (PAP), and human kallikrein 2 (hK2), are available for immunotherapy studies; however, their ability to induce strong T cell responses to date has not been demonstrated. The work outlined in this proposal is designed to identify biologically relevant, immunodominant antigens that have prostate restricted expression. The hypothesis is that transgenic mice expressing the human A2.1 major histocompatibility antigen, the most commonly expressed class I MHC molecule, can be used to screen for immunodominant antigens in vivo from cDNA libraries enriched for prostate cancer genes. The antigen discovery process utilizes known technology in a novel way to streamline the process of identifying immunologically important antigens. Although novel applications of technology are outlined as the primary approach to antigen identification, established alternatives are described to assure the technical success of the project. To accomplish the task of antigen identification, cDNA libraries enriched for expressed prostate tissue genes were developed and formal demonstration of the ability to use mRNA-pulsed dendritic cells as antigen presenting cells to immunize the transgenic A2.1 mice has been completed. Furthermore, a novel retroviral vector for expression of the enriched libraries has been engineered that not only will allow immunization from the libraries generated from tissue culture cell lines but also will provide a means of using libraries generated from primary prostate cancer tissue for antigen identification.

Body of Progress Report

Our effort to identify immunodominant antigens with prostate specificity are proceeding rapidly, but changes in strategies have been initiated because of new scientific developments and our preliminary observations. The modifications to the experimental approach and the reasons for the change are described below. Once validated, we believe that the new approach will provide a basis for efficient identification of antigens from all tissue types including primary prostate cancer from clinical specimens. We outline below the approved tasks and our progress toward accomplishing each.

Task 1. To effectively immunize major histocompatibility antigen (MHC) A2 transgenic mice against prostate cancer cells.

The procedure for effective immunization was identified. The initial plan was to use transgenic mice expressing the human MHC haplotype, A2.1, to identify immunodominant antigens. Since the human MHC, A2.1, is expressed in genetically modified mice and mouse T cells respond to the A2.1 peptide antigen complex, CD8 signals were not initiated.. The original working hypothesis was that the absence of CD8 signaling would be beneficial, since only strong antigenic determinants would be recognized by mouse T lymphocytes.[1] Immunization with LNCaP cells revealed that the A2.1 transgenic mice did not respond adequately. To over come this problem, we obtained another transgenic mouse line that expresses a modified A2.1 protein, A2.1K^b. The A2.1K^b protein has the alpha 3 domain from mice, which allows murine CD8 signaling but does not change the specificity of the peptide:MHC

interaction.[2,3] This mouse line has provided the necessary sensitivity for identification of reactive T lymphocytes (see below).

Task 1a. Identify prostate cancer xenografts that express A2 MHC but that share few or no MHC at the B and C loci. (months 1-12)

We tested MHC expression on prostate cell lines and xenografts available to us as well as other pertinent cell lines. Table 1 below shows the results. These data show that LNCaP and PC3 express A2. Only one xenograft, LUCaP-35, was observed to express A2. We have obtained LUCaP-35 from Dr. Robert Vessella at the University of Washington. These cells will be used to test reactive T cells identified by LNCaP immunization and at a later date for the identification of unique antigens. We are in the process of transfecting each prostate cell line and cell lines of non-prostate origin with A2 in order to have reagents available to validate the specificity of T cells generated by our immunization process.

Table 1. Cell Lines Expressing MHC Class 1 Allele, A2

Xenograft or Cell Line	A2 Expression
JY	+
Jurkat	+
T24	-
253J	-
DU145	-
PC3	+
LNCaP	+
ALVA-31	-
LUCaP-35	+

Task 1b. Identify from among the A2 MHC-expressing prostate cancer xenografts those that also express prostate specific antigen, prostate specific membrane antigen, and human kallikrein 2 (months 1-12)

This task will not be necessary because of the new strategy that will be used to identify novel antigens (see Conclusions below). By using dendritic cells as antigen presenting cells, we can protein pulse dendritic cells and use them as targets for isolated CTL. This is a more efficient approach.

Task 1c. Verify that the prostate cancer xenografts are capable of immunizing A2.1K^b transgenic mice (months 2-13)

This task has been delayed and will be pursued after completion of the identification of antigens from LNCaP immunization.

Task 1d. Immunize A2.1K^b MHC transgenic mice for the isolation of MHC A2.1 restricted cytotoxic T lymphocytes (months 3-18)

Immunization of A2.1K^b mice has been accomplished. As indicated above, immunization of A2.1 mice was not successful, but with the addition of the murine CD8 binding domain to the A2.1 molecule, the anticipated immunization profile was obtained. Immunization of A2.1K^b mice with either a dendritic

cell/LNCaP conjugate as described by Celluzzi and Falo[4] or LNCaP cells alone was tested for the ability to activate CTL (Figure 1). 7-10 days later spleen cells were isolated and co-cultured with LNCaP cells in vitro in a standard CTL stimulation assay.[5] Four days after initiation of culture viable lymphocytes were isolated by discontinuous density centrifugation and tested for lytic activity against LNCaP cells and a control non-prostate A2-expressing line JY. The data show that immunization with LNCaP cells was not effective in inducing CTL activity (data not shown). In contrast, the LNCaP/dendritic cell conjugate effectively immunized the A2.1K^b mice. These data showed that dendritic cells were strong priming agents as had been previously described in other model systems.[4] Evaluation of the reactivity of the activated CTL showed that the predominant antigen was allogenic MHC. Thus we have further modified the protocol to exclude whole cells and are using mRNA from tumor cells. To validate the system, we immunized mice with an adenovirus carrying the gene for ovalbumen, a strong inducer of CTL in C57BL/6 mice. In this study spleen cells were co-cultured with dendritic cells pulsed with antigen. The data show that dendritic cell co-culture with primed spleen cells was an effective means of stimulating the expansion of ovalbumen specific CTL (Figure 2). In the next experiment A2.1K^b mice were immunized with an adenovirus carrying the gene for PSA (Figure 3). In these experiments the in vitro co-culture was with cells infected with pox virus carrying PSA as described for other models.[5] The pox virus was used so that only PSA specific antigen recognition was possible. Since adenovirus carrying the PSA gene was used for the priming event, it could not be used to expand the CTL in vitro because of the presence of virus specific CTL. While the background lysis is higher than desired, the data show significant PSA-specific lytic activity.

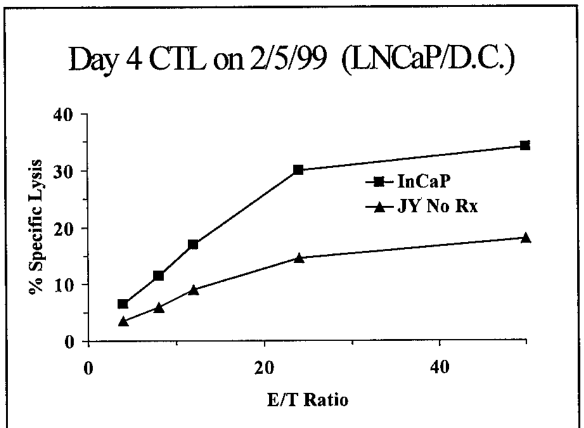

Figure 1. Activation of CTL activity using dendritic cell/LNCaP conjugates as priming agents. Immunization with LNCaP alone did not induce lytic activity.

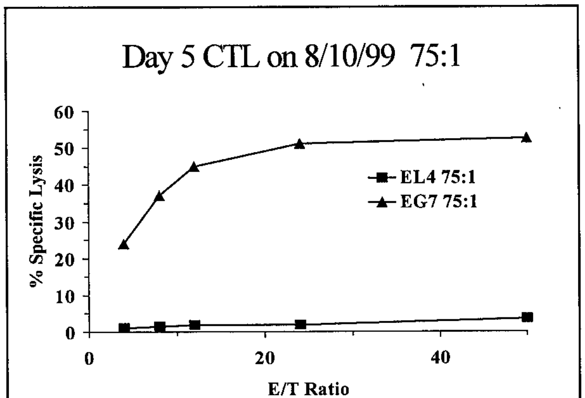

Figure 2. Evaluation of the most effective ratio of dendritic cells for co-culture conditions in secondary in vitro CTL expansion assays. Show is the 75:1 (responder to dendritic cell) ratio. Others tested but not shown are 10:1 and 25:1.

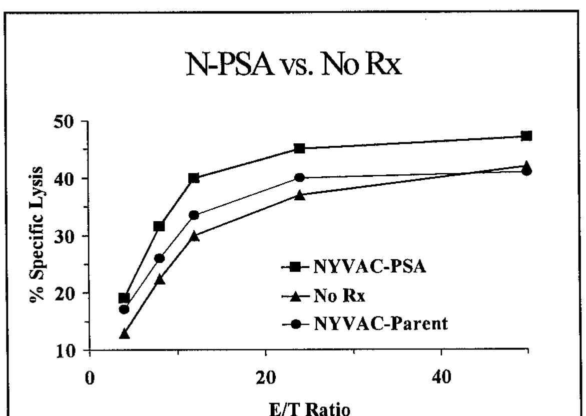

Figure 3. Utility of dendritic cells as priming agents for PSA in A2.1K^b mice. In this study, dendritic cells expressing PSA delivered by a pox virus (NYVAC-PSA) were used in the secondary expansion culture at a 75:1 ratio.

These data show that the use of dendritic cells for the in vivo priming of mice and also for the purpose of expanding CTL in vitro is the preferred method for activating CTL.

Task 2. To identify prostate specific MHC A2 restricted cytotoxic T lymphocyte clones

- in vitro expansion and cloning of A2 restricted cytotoxic T lymphocytes (months 3-12)
- characterization of the specificity of the cloned cytotoxic T lymphocyte cell lines (months 6-18)

These tasks have been delayed but should be completed this year

Task 3. To identify and purify MHC A2 binding peptides recognized by the prostate specific cytotoxic T lymphocytes.

- prepare MHC A2 expressing prostate cancers for extraction of A2 binding peptides (months 1-30)
- extract and concentrate MHC A2 binding peptides (months 12-30)
- separate MHC A2 binding peptides by high pressure liquid chromatography and test peptide fractions for recognition by the prostate specific cytotoxic T lymphocytes (months 12-30)
- purify and sequence active peptides by tandem mass spectrometry
- synthesize a panel of potentially active peptides to confirm appropriate amino acid sequence. (months 18-30)
- gene bank search for identification of proteins containing the identified sequence (months 24-30)

The work outlined in Task 3 will not be pursued. Instead, a molecular approach to the identification of antigens recognized by the cloned CTL will be followed (see Conclusions below).

Task 4. Biological characterization of the identified peptides.

- verification of the activation of peptide responsive T lymphocytes in MHC A2 transgenic mice (months 24-30)
- testing of the ability of lymphocytes from prostate cancer patients to respond to the identified peptide (months (24-30)
- testing of the ability of cytotoxic T lymphocytes responding to the identified peptide to mediate antitumor activity.(months 24-30)

The work described in task 4 will not be pursued. Instead, a molecular approach will be used to identify the protein (see Conclusions below).

Key Research Accomplishments

- Identification of an appropriate immunization method that will allow use of primary prostate tissue for immunization
- Determination that A2.1K^b mice respond to prostate antigens.
- Development of a vector capable of incorporating a subtracted prostate library to immunize mice
- Development of culture conditions for dendritic cells
- Development of immunization schedule for immunizing mice with RNA pulsed dendritic cells
- Development of subtracted prostate cancer library

Reportable Outcomes: none to date

Revised Experimental Design and Accomplishments toward Achieving the Objectives. The objective of the studies outlined herein is to identify prostate specific antigens that induce strong T cell responses. The studies propose to identify immunodominant prostate specific antigens by combining a molecular approach to generate a cDNA library enriched for prostate specific genes with immunization of transgenic mice to allow in vivo biological selection of immunodominant antigens. This approach offers advantages over previous antigen discovery methods including enrichment of prostate associated antigens for CTL activation and the ability to use mRNA isolated from microdissected primary tumor foci to generate prostate antigen discovery libraries. The process includes 4 distinct steps: (a) isolation a cDNA library enriched for genes expressed by prostate cancer cells, (b) generation of dendritic cells expressing peptides encoded by the cDNA library, and isolation of CTL reactive with strong antigenic

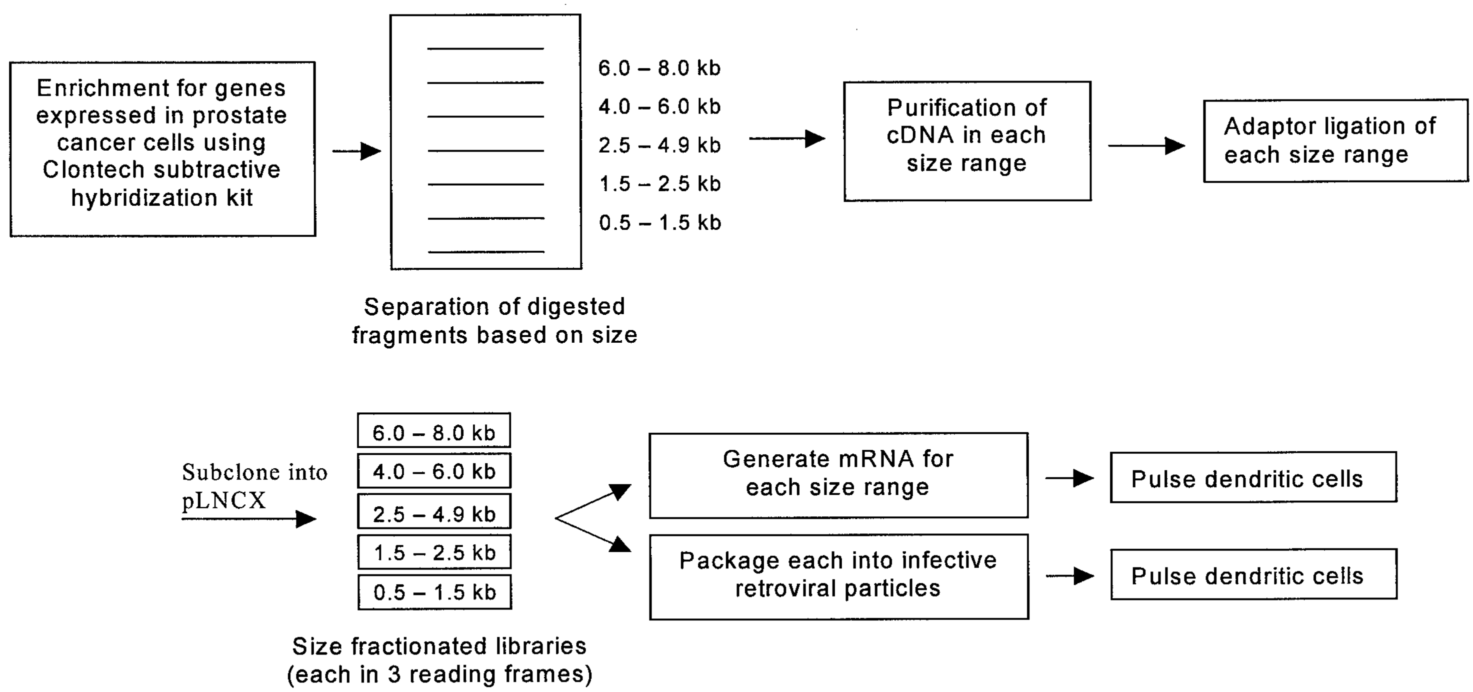

Figure 4. Library Development and Generation of Dendritic Cells Expressing Prostate-Enriched Antigens

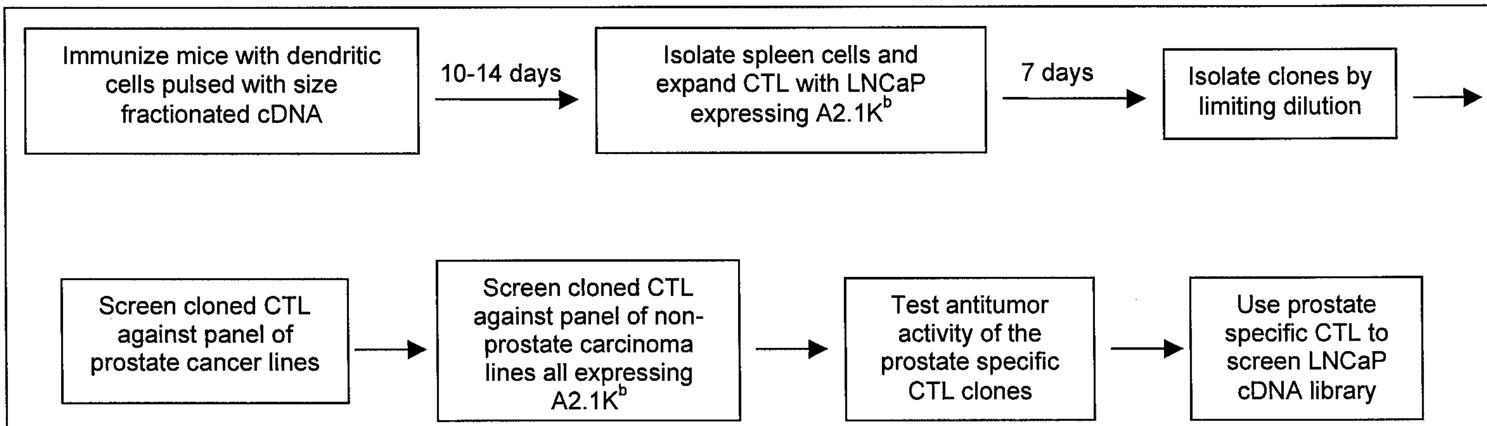

Figure 5. Generation of Antigen-Specific CTL Clones and Identification of Genes Coding for the Stimulating Antigen

determinants expressed by prostate cancer cells, (c) screening a cDNA library to identify the antigen recognized by CTL clone, and (d) verification of the immunogenicity of the identified gene product. A schematic of the antigen discovery process is shown in Figures 4 & 5.

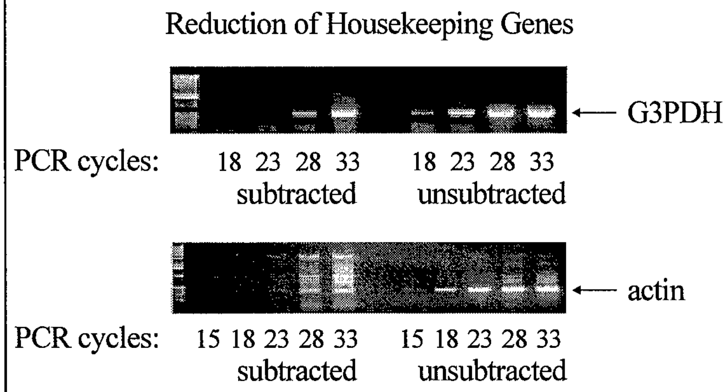

Figure 6. cDNA library enriched for genes expressed in the prostate cancer cell line, LNCaP. Shared "housekeeping" genes were subtracted with a cDNA library from the bladder carcinoma line, T24.

Generation of a cDNA library enriched for genes expressed by prostate cancer cells. Because effective immunotherapy depends on an antigen that is both immunodominant and prostate specific, we have used a molecular technique to generate a library that is enriched for prostate-specific genes. This was performed to reduce the proportion of genes that are present in all cells including prostate cancer cells such as glyceraldehyde 3'-phosphate dehydrogenase (G3PDH) and actin, commonly referred to as "house-keeping genes". To accomplish this, two libraries can effectively be "subtracted", with common genes (such as actin and G3PDH) being reduced or removed. For our second library, which was used to subtract housekeeping genes from the prostate library, we chose T24 bladder carcinoma epithelial cells. To enrich for prostate genes by subtraction, we first generated cDNA libraries from both LNCaP and T24 carcinoma cell lines using a proprietary library construction strategy to ensure full-length cDNA representation (Clontech SMART^{tm} kit). Subsequent *Rsa I* digestion and subtraction to enhance for prostate-specific genes was performed by representational difference analysis with suppression PCR (Clontech PCR Select^{tm}). The resultant subtracted library reveals a dramatic reduction of common genes (such as actin and G3PDH) but retains the presence of PSA (data not shown), suggesting enrichment of prostate specific genes (Figure 6). The Clontech library will be fractionated based on size, and subcloned into the modified pLNCX retroviral vector (see below). As an alternative to the Clontech subtracted library, we are collaborating with Dr. Bento Soares (Department of Pediatrics, University of Iowa), who is noted for his full-length cDNA libraries used in the CGAP program. Dr. Soares currently is generating a full-length cDNA library from LNCaP cells. Bento also will use T24 bladder cancer cells for subtraction. This library also will be

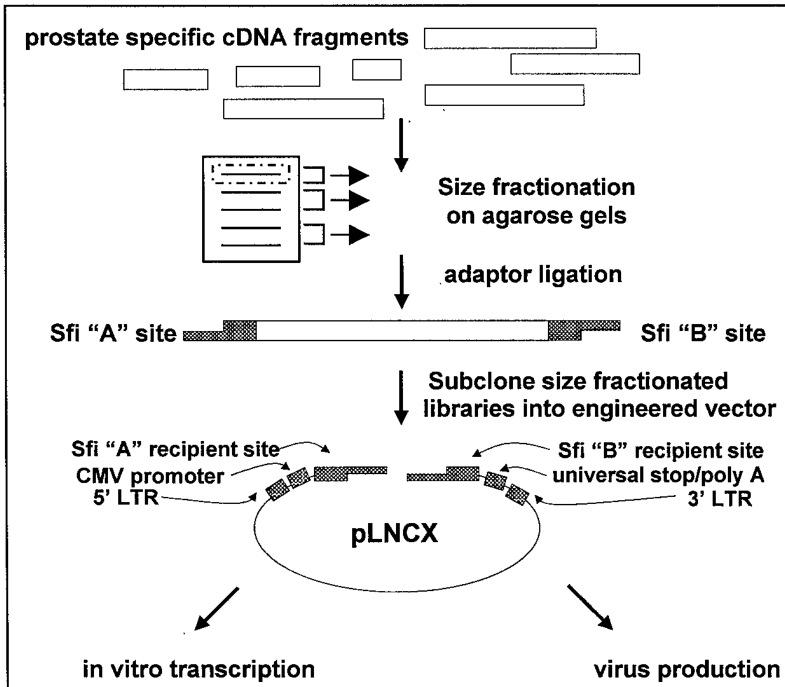

Figure 7. Engineered pLNCX vector used for generation of prostate enriched cDNA library.

subcloned into the modified pLNCX vector.

Novel Expression Vector for library generation. The design and construction of the new retroviral vector is shown in Figure 7. To generate a library capable of inducing a strong immune response, it must be expressed in manner that allows normal antigen presentation to occur. This can be done in either of two ways, both of which will be pursued with the multifunctional vector into which the library will be inserted. The first utilizes in vitro transcription of RNA, which is then pulsed into mouse dendritic cells. The preliminary data outlined below demonstrate that RNA-pulsed dendritic cells induce a robust CTL response to genes encoded by the RNA. The second approach utilizes the vector as the basis of a retrovirus system to produce a pool of retroviral particles that can efficiently infect dendritic cells, produce the encoded protein and activate CTL responses.[16] Since no currently available vector contains the required components, the pLNCX vector was modified to include the necessary features, which are shown in Figure 7. The backbone of the vector is the commercially available retroviral vector pLNCX (Clontech). This contains 5' and 3' long terminal repeat (LTR) sequences to allow efficient retroviral packaging when transfected into an appropriate packaging cell line (e.g. EcoPac 293, Clontech). The early cytomegalovirus (CMV) promoter drives high level transcription in mammalian cells. Some of the features we have engineered include the introduction of asymmetric, non-palindromic restriction sites (Sfi "A" and Sfi "B") to allow efficient, directional subcloning of the subtracted library. As designed, only library inserts with ligated adaptors will allow circularization and re-ligation of the vector for efficient transformation. Addition of an upstream T7 site, a consensus Kozak sequence, and a downstream poly A tract allow for efficient production and stabilization of in vitro produced RNA transcripts and subsequent high level translation. Three adaptor sets have been designed to allow insertion of the subtracted library into the Sfi "A" and Sfi "B" sites. These have been designed to stagger the start codon by one, two or three nucleotides, allowing all cDNAs in the library to be expressed in all three reading frames.

The retroviral library also will be used if higher efficiency gene transfer is required. Once prostate specific cloned T cell lines are identified, they will be used to screen cells that have been infected with the retrovirus library to isolate the appropriate gene sequence. Vector sequence that flanks the inserted cDNA will serve as priming sites to allow PCR amplification of the unique cDNA insert encoding the immunodominant antigen. To date, the subtraction process has been successfully completed, the vector has been constructed, and adaptors are available. We are currently pursuing adaptor ligation with size-fractionated sequences, and subcloning the library into the vector for both in vitro RNA production as well as generation of the retroviral library. Size fractionation will assure optimal representation of varying cDNA sizes by diminishing cloning bias in the library. Use of the completed library for in vitro transcription of RNA, pulsing of dendritic cells and immunization is described below. This molecular approach is being validated with tissue culture LNCaP cells. Once novel antigens are isolated from LNCaP cells, further studies will be performed with RNA from freshly isolated, microdissected prostate cancer tissue.

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